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The Role of PSR in Zebrafish (*Danio rerio*) at Early Embryonic Development

Wan-Lun Taung, Jen-Leih Wu and Jiann-Ruey Hong

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Abstract

During development, the role of the phosphatidylserine receptor (PSR) in the professional removal of apoptotic cells that have died is few understood. Programmed cell death (PCD) began during the shield stage (5.4 hpf), with dead cells being engulfed by a neighboring cell that showed a normal-looking nucleus and the nuclear condensation multi-micronuclei of an apoptotic cell. Recently, in the zebrafish model system, PS receptor played a new role on corpse cellular cleaning for further normal development during early embryonic development, which also correlated with tissues' or organs' complete development and organogenesis. In the present, we summary new story that a transcriptional factor, YY1a, in the upstream of PSR is how to regulate PS receptor expression that linked to function of PSR-phagocyte mediated apoptotic cell engulfment during development, especially the development of organs such as the brain and heart. YY1a/PSR-mediated engulfing system may involve in diseases and therapy. This engulfing system may provide new insight into phosphatidylserine receptor how to dynamic interaction with apoptotic cell during priming programmed cell death.

Keywords: programmed cell death, apoptosis, phosphatidylserine receptor, early embryonic development, brain, *in vivo* rescued

1. Introduction

Apoptotic cell death occurs by a mechanism that is conserved from nematodes to humans [1]. *In vivo*, the typical fate for apoptotic cells is rapid engulfment and degradation by phagocytes [2]. Among higher organisms, the removal of apoptotic cells by phagocytes suppresses inflammation, modulates the macrophage-directed deletion of host cells, and critically

regulates the immune response of an individual [3]. Cell death that is morphologically and genetically distinct from apoptosis is strongly implicated in some human diseases [1].

For vertebrates, the phagocyte engages the dying cells through specific receptors that include the phosphatidylserine receptor (PSR) [4–6], complement receptors 3 and 4, the ABC1 transporter, and members of the scavenger-receptor family [7]. Recently, T-cell immunoglobulin mucin protein 4 (TIM4), a phosphatidylserine (PtdSer)-binding receptor, mediates the phagocytosis of apoptotic cells. TIM4 engages integrins as co-receptors to evoke the signal transduction needed to internalize PtdSer-bearing targets such as apoptotic cells [8]. And PSR-1 enriches and clusters around apoptotic cells during apoptosis. These results establish that PSR-1 is a conserved, phosphatidylserine-recognizing phagocyte receptor [9].

For non-vertebrate systems such as the nematode *Caenorhabditis elegans* [10, 11] and *Drosophila melanogaster* [12], it illustrates the power of using genetically tractable systems to identify necessary phagocytic genes. Major efforts to understand crucial pathways that mediate programmed cell death (PCD) have also led to the genetic and molecular characterization of a number of genes involved in the recognition and engulfment mechanisms of cells among invertebrates [10, 13–15]. For *Caenorhabditis elegans*, it is important to recognize that phagocytosis is performed by cells that are non-specific phagocytes rather than by specialized phagocytes such as macrophages, as tends to be the case in *Drosophila melanogaster* [3].

For lower vertebrate systems such as the zebrafish, the cell corpses generated developmentally are quickly removed, although which specific type(s) of engulfment genes are involved still remain largely unknown. Little is known regarding the molecular mechanisms by which the resulting (cell) corpses are eliminated and the clearance of defective events for zebrafish. The zebrafish PSR-engulfing receptor was cloned (zfpsr) by Hong et al. [16], and its nucleotide sequence, was compared with corresponding sequences in *Drosophila melanogaster* (76% comprising identity), human (74%), mouse (72%), and *Caenorhabditis elegans* (60%). The PSR receptor contained a JmjC domain (residues 143–206), and localization was labeled in chromosome 3 (GCF-000002035.6; accession number: NC-007114.7). Very recently, the PSR gene was regulated by YY1a transfection factor [17].

2. What is programmed cell death?

The concept of natural cell death can go back to 1842 [18]. Karl Vogt found that midwife toad eliminates notochord and forms vertebrae during metamorphosis. The death of this cell depends on the regulation of endogenous genes and hence giving rise to the term programmed cell death (PCD). Now the PCD has generated a new concept and new clarification.

2.1. Type I cell death: apoptosis

Apoptosis, a type of programmed cell death, is a mechanism in developing embryos that removes damaged cells without impairing the overall development of normal tissues [19]. Controlling factors of apoptosis include the B-cell lymphoma 2 (Bcl-2) protein family that

inhibits apoptosis, the Bcl-2-associated X protein (Bax) protein that promotes apoptosis, and the aspartate specific cysteine protease (Caspase) family of proteins [20, 21]. Regulation of apoptosis includes regulation both inside and outside. Among them, the activation of the external is mainly when the death ligand on the cell membrane binds with the death receptor, and the apoptosis pathway is activated, which in turn activates the downstream Caspase-8 and the downstream Caspase-3 to promote apoptosis. Internal activation is mediated mainly by endogenous stimuli such as DNA damage, Bax, and Bcl-2 homologous antagonist killer protein (Bak) that cause the activation of pro-apoptotic Bcl-2 family in the mitochondrial outer membrane, resulting in grain line and then release of cytochrome C to combine with apoptotic protease activating factor 1 (Apaf-1) to form apoptotic body (apoptosome), which in turn activates downstream Caspase-9 and downstream Caspase-3 to promote cell apoptosis [21].

2.2. Type II cell death: autophagy

Autophagy is a catabolic process that involves the degradation of cytoplasmic components, protein aggregates, and organelles through the formation of autophagosomes, which are degraded by fusion with lysosomes. The autophagy process has been extensively well studied in the response to starvation of *Saccharomyces cerevisiae*, in which it protects cells from death by recycling cell contents. Autophagy depends on a large group of evolutionarily conserved autophagy-related genes (ATG) [22]. On the other hand, the protective, pro-survival function of autophagy, silencing, and deletion of ATG genes that resulted in accelerated cell death [23, 24] was proposed. However, in certain scenarios, it has been suggested that severely triggered autophagy process can lead to or contribute to cell death.

2.3. Type III cell death: regulated necrosis

In the early stage, necrosis was regarded as an unregulated mode of cell death that was caused by overwhelming trauma. However, many recent studies indicate the existence of several modes of regulated necrosis [25]. Necrosis is characterized by swelling of organelles and cells, rupture of the plasma membrane, and release of the intracellular contents. Different modes of regulated necrosis share common morphological features. Then, the best well-studied form of regulated necrosis, also called necroptosis, is a type of necrotic cell death that depends on receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and/or RIPK3 [25–27]. Additional necrosis is induced by different stimuli, but it remains to be shown that these actually involve different mechanisms for programmed necrosis [25].

3. The zebrafish development in the early stage

Zebrafish embryos develop from the one-cell stage after fertilization. The cells are then split in multiples to blastocysts' stage (see **Figure 1**); then enters the gastrula stage. During this period, embryonic cells begin to differentiate into three germ layers via apoptosis. Each germ layer will differentiate into specific organs such as endoderm cells that differentiate into respiratory and gastrointestinal epithelial linings and include glandular cells such as

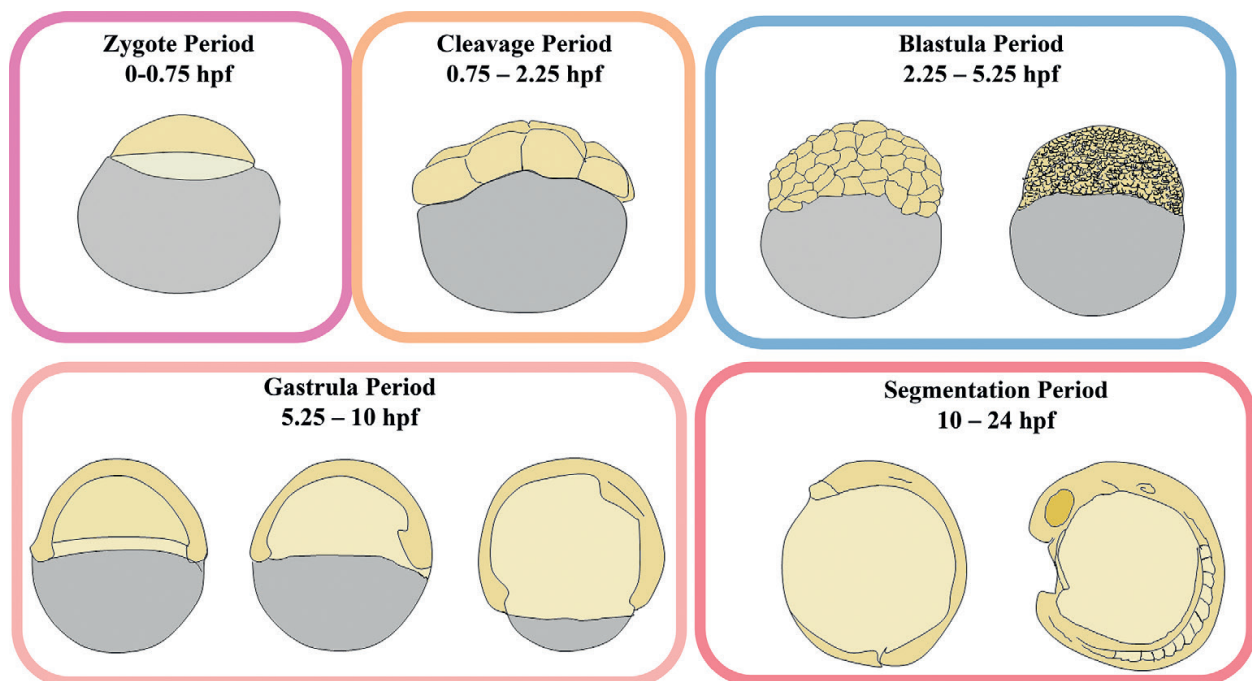


Figure 1. A scheme of zebrafish embryonic development stages was shown. After fertilization, one-cell formation in animal pole is for about 30 min and then begins dividing into two cells (0.5–1.0 hpf) and entering into blastula stage (2.25–5.25 hpf), gastrula stage (5.25–10 hpf), and segmentation stage (10–24 hpf) that finally can hatch out between 48 and 72 hpf. In the right time in the gastrula stage at 5.4–6.0 hpf, the programmed cell death is turned on and the professional engulfing corpses system by PSR works for smoothing embryonic development.

the liver and pancreas of the relevant organs. The mesoderm will differentiate into smooth muscle layers, smooth muscle coats, connective tissues, and blood vessels that supply these organs. It is also a source of blood cells, bone marrow, bone, striated muscles, reproductive, and excretory organs. This period of cell differentiation behavior and follow-up organ development are closely related. Then enter the segmentation, pharynx, and incubation periods (Figure 1) [16].

4. The role of apoptosis in early developmental stage in zebrafish

In the zebrafish system, apoptosis is regulated by Noxa, which is a novel regulator of early mitosis before the 75% epiboly stage when it translates into a key mediator of apoptosis in subsequent embryogenesis [28]. PCD turned on was observed by electron microscopy and the earliest onset of programmed cell death in zebrafish embryos was about 6 h after fertilization, and typical apoptosis was observed in zebrafish embryos in the gastrulation stage [16]. In addition, 12–96 h after fertilization, signals of apoptotic cells can be detected in the nervous system and sensory organs such as the retina, ear, and olfactory organs [16].

5. After PCD starting: engulfing of corpses death cell by professional system, PSR and others

The *Caenorhabditis elegans* system is known to have a nonspecific phagocytic system that regulates cell death through the use of cell death abnormality protein 1 (CED-1) to identify cellular debris and transmit related messages and another group of cell death. The system of abnormality protein 2 (CED-2) affects the cytoskeleton, causing the cell membrane to collapse, while activating the relevant GTP synthetase to generate sufficient energy to provide cellular pattern changes. By this system, Wang et al. confirmed that PSR-1 of the nematode activates CED-2, further enabling phagocytes to recognize apoptotic bodies and using enzyme immunoassay to prove that PSR-1 itself can interact with phosphatidylserine [11].

Proliferation of programmed cell death (PCD) is caused by the condensation of chromatin DNA, which leads to the cleavage of DNA in the cell and the cleavage of the membrane by the nuclear pore to form nuclear fragments. In the process of programmed death, dehydration will continue, the cytoplasm is concentrated, resulting in vesicle-like cell membrane, and cell size decreases. Apoptotic cells produce nuclear fragmentation and form chromatin masses (nuclear fragments), resulting in sprouting of cells, the formation of a spherical bulge, and other means of cell protrusions, eventually resulting in a range of sizes, including the cytoplasm, organelles and nuclear debris, and other small bodies, which can be called apoptotic bodies [29]. In general, there are three main components of the phospholipids in the cell membrane, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) located on the inner side of the cell membrane [30]. When cells undergo apoptosis, the phospholipid structure inside the cell membrane moves to the outside of the cell membrane, at which time the PS located on the surface of the cell membrane becomes an important marker of the phagocytic apoptotic bodies [31, 32]. Scientists with competing PS analogs competed for the ability to inhibit PS clearance of apoptotic cells, but failed to do so for other phospholipids, such as PC, confirming that phospholipid serine (PS) can be specifically affected by identification [32]. Fadok et al. further induced macrophages to produce PSR antibody mAb 217 with transforming growth factor beta (TGF-beta) and beta-glucan [4]. mAb 217 can be used to calibrate phagocytes with the ability to recognize, and vice versa phagocytes without identifying apoptotic bodies, so that the monoclonal antibody can identify possible target proteins and purify the deglycosylation to obtain the target protein of 48 kDa, PSR [4]. However, previous studies on phospholipid serine receptors (PSRs) have shown a nuclear localization of phospholipid serine receptors mainly in vertebrate cell lines and invertebrates [33]. Then, by comparing the results of amino acid sequence-predicting phospholipids, serine receptors may have DNA binding structures and Jumonji C domain (JmjC domain); the results of this comparison in different species, including from hydra to humans, all have a certain conservative [33, 34]. In addition, nematodes [4, 11], fruit flies [4], zebrafish [16], and mouse [6] also exist in the PS receptor.

6. The role of zebrafish PSR in early embryonic development

In the zebrafish system, Hong et al. [16] compared the PSR genes of different species in the cDNA library to further identify the *zfpsr* homology gene of PSR in zebrafish. After the zebrafish embryos were fertilized, by in situ hybridization, to observe the *zfpsr* development in whole embryos, *zfpsr* mRNA was found in one-cell embryo and then displayed in different tissues and organs as time progressed [16]. At 24 h (see **Figure 2**) after fertilization, the head, eyes, body axis, and chordal can be observed in the performance of the gene. After 3 days of fertilization, *zfpsr* mRNA can be observed in specific organs such as the heart, trunk, kidney, and other organs in the development of the gastrointestinal tract and is the predominant organ of *zfpsr*. Then, the *zfpsr* gene is silenced (loss of function by a morpholino), resulting in the accumulation of apoptotic cells in zebrafish development, further resulting in embryonic brain, heart, chordate, somite dysplasia. In severely deficient embryos, the brain is impaired, there incomplete development of the posterior nodules, and an inability to hatch. Slightly deficient embryos developed in the heart and in the absence of the apical development, including the heart chambers and the aorta. Large veins underwent incomplete development. The severely affected embryos accumulated large amounts of cellular debris 12 h after fertilization and died 3 days after fertilization.

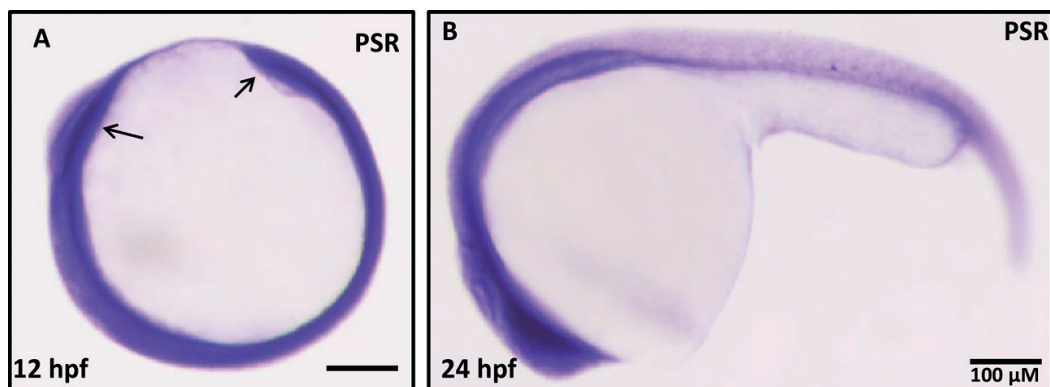


Figure 2. Identification of PSR expression pattern during early zebrafish embryonic development. (A) PSRs are expressed in the whole embryo including the ectoderm, mesoderm, and endoderm, especially with the major location for PSR being within the brain region and the posterior of the embryo (indicated by arrows) at 12 hpf and (B) at 24 hpf, PSR is expressed in the whole notochord and distributed in the trunk, brain, and the eyes. Scale bar = 100 μ m.

7. Why PSR is important in zebrafish?

Previous studies indicated that the environmental stress in zebrafish embryos may be related to the regulation of mitogen-activated protein kinase. And mitogen-activated protein kinases have been shown to regulate cellular migration in previous studies [35, 36], suggesting that adversity within the embryo may affect cell migration. In our laboratory, it was observed that the zebrafish embryos after PSR knockout were observed in the early stage of embryonic development of the intestine, and the laryngeal phenomenon of outsourcing was observed

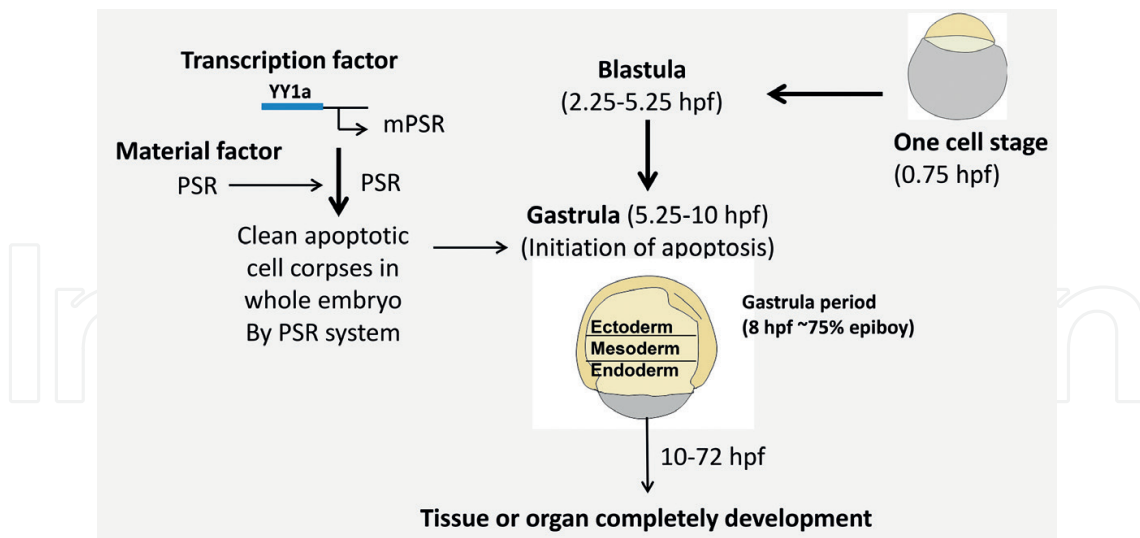


Figure 3. A summary of the zebrafish PSR story during the entire development. The role of PSR function, especially in the gastrula stage of zebrafish embryo, is very important to the event of epiboly. The programmed cell death star via apoptosis may be at 75% epiboly stage as compared to PSR knockdown control. During this stage, the cell death should be cleared out by the engulfing system that may be working through the PSR engulfing system. If not, dead cells will accumulate and cause normal cell migration. At the same time, the accumulated corpus cell can enhance environmental stress via reactive oxygen species (ROS) production in the whole embryo. Finally, these reasons may cause the phenotype of cell fate disruption within three germ layers and continue till late development stage.

(see **Figure 3**), and the differentiation of the three germ layers was not clear. Previous studies have also confirmed that the PSR gene may be involved in the clearance of dead cells [16]. Our study delayed the migration of PSR cells after they were deactivated, which might be due to the unclear death cells, which blocked the movement of the whole cell layer and delayed the development of zebrafish embryos in the primitive intestine. However, the mechanism is still not clear. By virtue of the early embryonic development of endoderm-labeled embryonic forerunner cells, progenitor cells are severely damaged after PSR loss of function and lead to disruption of the differentiation of the cell cycle to cell migration. It is concluded that the PSR gene affects the migration and differentiation of cells in the embryonic development of the intestine, leading to the inability of cells to determine the fate of the cells in the early development of the embryo and disrupting the distribution of the germinal layers. For new supporting case, such as skeletal muscle arises from the fusion of precursor myoblasts into multinucleated myofibers. A new report by Hochreiter-Hufford et al. [37] identifies apoptotic cells as a new type of cue that induces signaling via the phosphatidylserine receptor BAI1 to promote fusion of healthy myoblasts, with important implications for muscle development and repair.

8. Conclusion and perspectives

In gastrula stages of zebrafish early embryo development, germ layer differentiation is quite important. This chapter is about the formation of follow-up organs. Differentiation of the germ layer requires apoptosis to assist in participation. According to the results of our laboratory research, the loss of function of the PSR gene by knockout approach can result in the

failure of early gastrula-stage germ layer differentiation, which in turn led to the increase of oxidative stress in zebrafish embryos, triggering severe apoptosis (unpublished data). Then, PSR knockout induced some damaged organs and tissues which was observed at 72 hpf that was also linked to cardiovascular dysplasia and swimming behavior. A summary of the abovementioned suggests that PSR gene on embryonic development and organ development has a certain impact, which is strongly associated with cardiovascular dysplasia and brain development even on congenital diseases.

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